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Cover Photograph: White-topped Pitcher Plant (*Sarracenia leucophylla*)

Cover Photograph: Photo is courtesy of Bill Garland, U.S. Fish and Wildlife Service, Biologist, Anniston, Alabama. Photo was taken at Splinter Hill Bog in Baldwin County, Alabama. This carnivorous pitcher plant is a rare fire-dependent species of south Alabama longleaf pine savannas and bogs.

Editorial Comment:

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CONTENTS

ANALYSIS OF BACTERIAL DIVERSITY IN SOILS FROM BLOWING SPRING CAVE (LAUDERDALE COUNTY, AL)

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ABSTRACT

Subterranean environments have been shown to display relatively simple bacterial communities. More data on the diversity of bacteria present in these subterranean environments would help to establish a baseline for these ecosystems. Cave environments typically have limited light penetration and varied amounts of nutrient influx. Due to the limited availability of nutrition and the subsequent competition for these resources, these areas could reveal interesting bacterial community structures and interactions. Blowing Spring Cave (Lauderdale County, Alabama) was selected for this study and environmental DNA samples were isolated from soil samples taken from Blowing Spring Cave. A highly conserved region within the 16S rRNA gene was PCR amplified from these environmental DNA samples. 16S rRNA libraries representative of the soil bacterial community were generated and sequences from these libraries were compared to other 16S rRNA sequences available in GenBank. The most prevalent phylum in this cave soil sample was proteobacteria, with 43% of the sequences grouping into three sub-phyla: gamma-proteobacteria (23%), alpha-proteobacteria (19%), and beta-proteobacteria (1%). The remaining 57% separated into the following phyla: planctomycetales (12%), actinobacteria (9%), gemmatimonadetes (9%), firmicutes (5%), chloroflexi (5%), acidobacteria (4%), and 13% of unknown bacterial origin.

INTRODUCTION

Caves represent unique environments because they offer the ability to study organisms that have limited contact with the surface and photosynthetic carbon sources, and therefore represent areas with low nutrient availability (Engel et al., 2004; Simons et al., 2008). Blowing Spring Cave is a limestone formation created via carbonic acid and water erosion. A small creek runs through it year round and exits at the cave's

mouth. The hydrology of this environment can have a profound impact on the nutrition of the ecosystem and transient members of the consortia can be deposited from flush and sediment. The water flow also provides potential nutrient input from both dissolved organic carbon and coarse particulate organic matter (Simons et al., 2008). Limestone can also be rich in reduced sulfur, iron, and manganese compounds, which microbes can use via redox reactions (Northrup and Lavoie, 2001).

Some cave microbial communities have been shown to have low biotic composition complexity (Engel et al., 2004). Thus, it is easier to study community microbial interactions of this limited consortium. Soil bacterial concentrations are typically higher when associated with the soil interspaces found near the plant rhizosphere (Housman et al., 2007). Due to a lack of vegetation in caves, the bacterial community has evolved methods to obtain carbon from other sources, such as animal or microbial. It has been reported that there is an abundance of anaerobic activity in caves, where high concentrations of unoxidized organic matter are present (Hose et al., 2000). This unoxidized matter is possibly a carbon source that could compensate for the lack of vegetation.

Some studies have indicated chemolithotrophic bacteria activities in caves, causing biofilms and snottites, which form as extensions of microbial biofilms that coat the walls and ceilings of caves (Hose et al., 2000). Interesting microbial interactions may be present within these biologieal formations. According to Groth, et al., (1999) there is evidence to show that certain cave bacteria, namely actinobacteria, can produce chemicals with potential benefits to the field of medicine and industry, with (bbyproducts) useful as antibiotics and anticancer agents. Past reviews have shown that both detrital and productive systems exist in caves. Productive systems require an energy source, and in subterranean environments, the sources are typically of a geochemical nature in contrast to the photosynthetic sources of surface organisms (Stevens, 1997).

Caves are environments that can vary greatly from those found on the surface and are typically energetically stable with relatively high humidity and low constant temperature (Laiz et al., 1999). Subterranean microorganisms tend to have a low metabolic rate, creating a scenario that favors maintenance over growth (Stevens, 1997). Studies by Schabereiter-Gurtner et al. (2004) in two Spanish caves (Llonin and La Garma) and Engel et al. (2004) in Lower Kane Cave, Wyoming, both found proteobacteria to be the most common phylum present in those cave soils. Lower Kane Cave samples were taken from microbial mats and consisted of epsilon-protcobacteria (67%), gamma-proteobacteria (12%), beta-proteobacteria (12%), delta-proteobacteria (1%), acidobacteria (6%), and bacteroides and chlorobi (2%). The Spanish cave samples were taken from cave paintings and the surrounding rock surfaces and consisted of proteobacteria (41%), acidobacteria (16%), actinobacteria (20%), firmicutes (11%), cytophaga/flexibacter/bacteroides (6%), nitrospira (4%), chloroflexi (1%), and candidate WS3 division (1%). Distinct differences in sampling sites characterized the Engel et al. (2004) study, which pertained to a sulfuric acid pool and had lower diversity than that of the Schabereiter-Gurtner et al. (2004) study, which pertained to samples taken from cave paintings and their surroundings.

Research focusing on CAVEcave ecosystems has been rather limited and most

of the work concerning the bacterial diversity in caves has utilized conventional culturing methods. These methods are problematic in that they only allow detection of culturable bacteria, which typically represent less than 5% of the bacteria present in any given sample (Desai et al., 2006). In this study, we utilized polymerase chain reaction (PCR), cloning, and sequencing of a portion of the 16S rRNA gene to evaluate the bacterial community of the cave soil sample environment of Blowing Spring Cave (Lauderdale County, AL). These molecular techniques have a greater scope of bacterial identification than traditional culture techniques (Schabereiter-Gurtner et al., 2004).

MATERIALS AND METHODS

Site selection and sample collection

Blowing Spring Cave (Lauderdale County, AL., T2S, R7W, Section 19; 34.8644°, -87.3039°) is located on a 60 acre tract under the management of the Alabama Department of Conservation and Natural Resources (AL-DCNR). This location is a maternal gray bat colony site and is closed to the public, and therefore represents a It is relatively pristine cave system, with limited human contamination and due to the stream it represented a hydraulically unique sample site. Soil samples were collected in duplicate from two sites within the cave using sterile 20 mL centrifuge tubes. One soil sample from was taken from a rock shelf layer that was approximately one meter above the floor; while the second soil sample was taken directly from the cave floor. Samples sets were taken by scraping the top 2.5 cm of the soil, which displayed no observable layers at this sampling depth . Approximately 5 grams of soil per site was collected and stored at -20 °.

DNA isolation and 16S rRNA gene amplification

DNA was isolated from 0.25 g of soil from each of the two collection sites (sampled in duplicate for a total of four processed samples) using PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) with a modification of the published protocol (additional incubation at 70 °C for 10 min) . Standard concentrations (200ng/uL) of DNA taken from both cave shelf and cave floor were pooled (1:1 ratio), mixed and then used as template in subsequent PCRs. The final concentrations per reaction wereWAS: 3 units of hot start Taq polymerase, 200 uM dNTPs, 4mM $MgCl_2$, 0.4 mM 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 0.4 mM 1492R (5'-GGTTACCTTGTTACGACTT-3') 16S eubacterial primers (Dojka; et al, 1998), and 1uL of DNA template with final reaction volume of 25 µl. The PCR parameters were as follows: initial melt at 94°C for 12 min followed by 25 cycles of 94 °C for 30 sec, (SEC??) 50 °C for 30 sec (SEC??) and 72 °C for 60 sec (SEC??) and a final hold at 72°C for 5 min. The PCR amplified products (approximately 1,400 bp in length) were analyzed using agarose (0.9%) gel electrophoresis.

Clonal library construction

PCR products were cloned using the pCR8/GW/Topo® vector (Invitrogen Inc., Carlsbad, CA). Transformations were performed using One Shot® competent *E. coli* via manufacturer's protocol (Invitrogen Inc., Carlsbad, CA). The transformed cells were grown overnight at 37 °C on LB/agar plates containing 100 µg/ml of spectinomycin. A total of 120 colonies were selected for screening and plated onto LB/agar grid plates with spectinomycin (100ug/ml) and incubated at 37 °C overnight. Selected clones were grown in 5 ml LB broth with 50 µg/ml of spectinomycin and incubated overnight at 37 °C. Recombinant plasmids were isolated from the selected clones using a QIA prep® Spin Mini Prep Kit (Qiagen Inc., Valencia, CA) per manufacturer's protocol. The quantity and quality of each plasmid preparation was analyzed via spectrophotometer and then stored at 4 °C.

Sequencing

Sequencing was conducted using Beckman Coulter GenomeLab™ Dye Terminator Sequencing with Quick Start Kit (Beckman Coulter Inc., Fullerton, CA). Sequencing reactions were setup using the following reaction conditions: 100 ng of plasmid, 1.6 µM of 16S forward primer (27F), 2 µl of dye terminator cycle sequencing reaction (DTCS) mix and sterile water was added to bring the total volume to 10 µl. The cycling conditions were as follows: initial melt at 96 °C for 1 min, followed by 30 cycles of 96 °C for 20 sec, 50 °C for 20 sec, 60 °C for 4 min. Following the PCR amplification reaction, each reaction product was purified via ethanol precipitation. These PCR amplified products were then analyzed using the Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, CA).

Phylogenetic analysis

Analysis was performed using the MEGA 4.0 software (http://www.megasoftware.net). All sequences used in these analyses were a minimum of 500 base pairs after quality-based trimming on both the 5' and 3' ends. Results were compared to known sequences using the NCBI database Basic Local Alignment Search Tool (BLAST). Select sequence matches from BLAST (with similarities >97%) and those from the cave were aligned and trimmed with the CLUSTAL alignment tool. Phylogenetic trees based on 16S rRNA sequences were constructed using the Neighbor-Joining (NJ) method of analysis. Further, the reliability of each tree was estimated by conducting bootstrap sampling. Bootstrap values were based on 100 bootstrap replicates. Rarefaction data was calculated, based on phyla, using RarefactWin software (http://www.uga.edu/strata/software/) to determine the optimal number of sequences utilized for phylogenetic analysis in this study (i.e., coverage of the bacterial community at the sample site). GenBank accession numbers for the bacterial 16S rRNA gene sequences used for phylogenetic analysis and tree construction were as follows: Acidobacteria (DQ167080), Actinobacteria (AF409026), Actinobacteria (AY795640), Firmicutes (AJ544784), Gemmatimonadetes (AY795731), Alpha-proteobacteria (AY795711), Alpha-proteobacteria (AY371423), Alpha-proteobacteria (EF575560), Alpha-proteobacteria (EF018616), Alpha-proteobacteria (EU543062), Beta-

proteobacteria (AY123791), Gamma-proteobacteria (AY795728). GenBank accession numbers for non-redundant bacterial 16S rRNA gene sequences identified in this study are as follows: Uncultured baeterium – clone 7 (GU944688), Uneultured bacterium – clone 13 (GU944687), Uncultured baeterium – clone 26 (GU944686), Uneultured bacterium – clone 28 (GU944685), Uncultured bacterium – clone 44 (GU944684), Uncultured bacterium – clone 79 (GU944683), Uncultured bacterium – clone 89 (GU944682), Uncultured bacterium – elone 106 (GU944681), Uncultured bacterium – clone 117 (GU944680).

RESULTS

A total of 120 clones were sequenced in this study. Seventy eight of the clones met the minimum criteria for inelusion in the final analysis, which consisted of a 500 bp partial 16S rRNA sequenee from forward priming. Sixty one unique sequences were found of these 78 clones. Those with greater than 98% similarity were considered to be replieates. Sequences were grouped with their most closely related phyla/sub-phyla using NCBI database. MEGA 4.0 genetic analysis software suite was used to create a phylogenetie analysis of the 16S clones from Blowing Spring Cave, with bootstrapping analysis (i.e., 100 bootstrap replicates) of tree reliability (Fig. 1).

A total of seven known phyla were found, including proteobacteria. Two distinctive unknown groups were reported as unknown-1 and unknown-2 and were not categorized within any known phylum. The most prevalent phylum was proteobacteria with 43% of the total organisms found grouping into three sub-phyla: gamma-proteobacteria (23%), alpha-proteobacteria (19%), and beta-proteobacteria (1%). Remaining sequences separated into the following phyla: planetomycetales (12%), actinobacteria (9%), gemmatimonadetes (9%), firmieutes (5%), ehloroflexi (5%), and acidobaeteria (4%). Both unknown-1 and unknown-2 represented 6% of the total organisms (Fig. 1). Rarefaction data was completed using phyla organization to determine coverage of the baeterial diversity for the sample site. This rarefaction data indieated that the point where additional sampling produced no additional information about the number of phyla present in the Blowing Spring Cave soil samples (ie., optimal number of sequences utilized) was 60 sequences.

DISCUSSION

Phyla-based analysis is typieal of studies from extreme environments due to the rarity of individual speeies; therefore, the data from this study are presented at the level of phyla present in the soil samples. This limitation of speeifieity was due to method resolution and lack of species specificity in known 16S rRNA gene sequences. Phyla based analysis is typieal of studies from extreme and unique environments due to the rarity of individual speeies and this method allows better comparisons with both culture and molecular methods in the literature. Rarefaction analysis was used to determine phyla-based curves. While rarefaction was designed as a species richness tool, it has been reported

to be useful on higher taxonomic levels as well (Raup, 1975). Schabereiter-Gurtner et al. (2004) and Engel et al. (2004) both reported that the highest number of sequences at their study sites grouped with proteobacteria, as did 43% of sequences analyzed from Blowing Spring Cave (Table 1).

Comparisons of these samples indicated distinct differences between the subterranean and forest/agricultural environments; most notably the phylum acidobacteria, which was only reported in the cave samples, while the phylum verrucomierobia only showed up in the forest and agricultural samples (Upchurch et al, 2008). The phylum chloroflexi was also more prevalent in cave samples, only showing up at a low percentage (1%) in one of the five forest and agricultural samples. Rarefaction analysis was performed on the sample sites at Horse Shoe Bend, Lower Cane Cave, Llonin and La Garma Caves, and Blowing Spring Cave. The phyla-based diversity differed more within each particular environment than between the two types of environments (caves versus forest/agricultural). There were similarities between the caves and forest/agricultural environments, including proteobacteria being the most prevalent phylum.

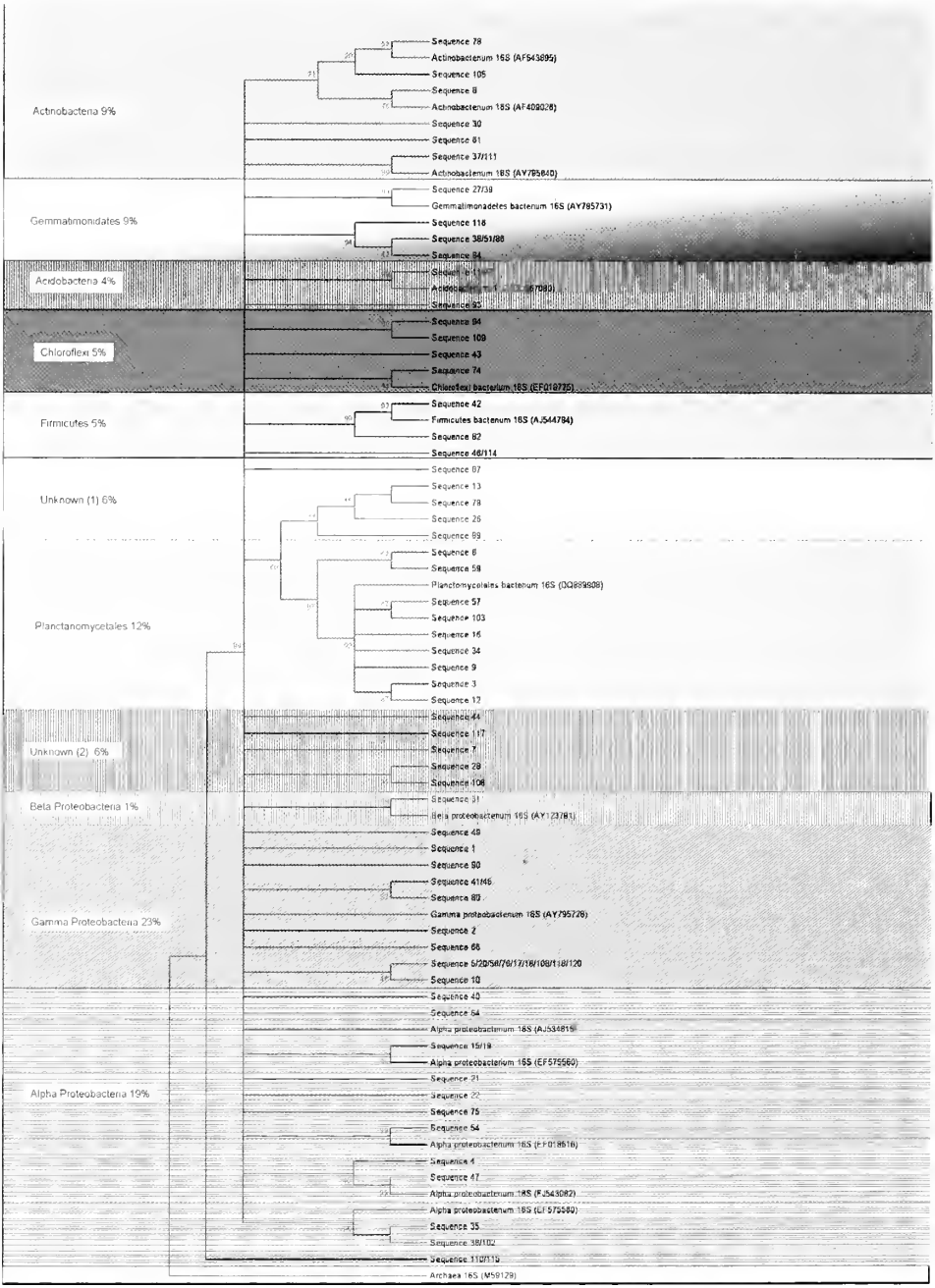

Figure 1. Phylogenetic diversity in Blowing Spring Cave as inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to each branch. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Phylogenetic analyses were conducted in MEGA4 software.

Table 1. Comparison of bacterial phyla present in three cave studies (Blowing Spring Cave, AL, Lower Cane Cave (Engel; et al, 2004), and Spanish Caves (Schabereiter et al., 2004)) and five forest & agricultural samples from Horse Shoe Bend, GA (Upchurch et al., 2008).

Bacteria Present	Cave Samples (% Present)			Forest and Agricultural Samples (% Present)					
	Blowing Springs Cave	Lower Cane Cave	Llonín and La Garma Caves	CT5	NT2	NT5	NF5	OF2	OF5
Acidobacteria	4	5.6	16.5						
Actinobacteria	9		20	4.3	10	13.8	10.1	1.1	8
Bacteroides		1.7	5.9	4.3	1.8	1.7	3	2.1	
Chlorofexi	5		1.2	1					
Firmicutes	5		10.6	1	1.8	0.9	4		1.1
Gemmatimonadetes	9			5.3	1.8	6.8	2	1.1	1.1
Nitrospira			3.5	1.1			1		1.1
Planctomycetales	12				7.3	0.8	2	1.1	3.4
Proteobacteria	43	92.7	41.1	33.3	33.9	51	32.3	43.9	32.1
Verrucomicrobia				4.3	6.4	5.1	4	13.1	3.4
WS3 division			1.2						
Unknown	13			45	37	19.9	41.6	37.6	49.8

Proteobacteria is a highly diverse phyla with five associated sub-phyla, three of which were found in the Blowing Spring Cave sample. One of the 16S rRNA sequences (sequence 31) displayed a homology of 98.2% to that of beta-proteobacteria genus *Nitrosospira,* which is a known ammonia oxidizing bacteria having the ability to transform ammonia (NH_3) to nitrite (NO_2^-) and can do these processes in relatively low concentrations (less than 1mM) of ammonia versus other genera, such as *Nitrosomonas,* which tend to thrive in higher concentrations (Taylor et al., 2006). The alpha-proteobacteria associated families of Rhodobacteraceae, Phyllobacteriaceae, and Rhizobiaceae were also present in the Blowing Spring Cave soil samples. Rhizobiaceae includes several species that are known to be associated with nitrogen fixation, as well as other environmental roles. (De Castro et al., 2008). Planctomycetales was the second highest represented phyla (12%) in Blowing Spring Cave soil samples, and according to Barion et al. (2007), it may have been one of the first groups to diverge in Domain Bacteria. Planctomycetales contain slow-growing bacteria and has been reported to have anaerobic ammonium-oxidizing species often found working in consortium with chloroflexi and proteobacteria (Liu et al., 2008), both of which were present in the Blowing Spring Cave soil samples. Actinobacteria, a phylum with known chitinolytic species, was also found in Blowing Spring Cave soil samples (9%). Some of these chitinolytic species can also produce antifungal agents specifically against organisms in *Fusarium*, the causative agent of white nose syndrome in bats in the northeastern United States (Yasir et al., 2009). The phylum gemmatimonadetes is a newly recognized phylum and was found to be present in Blowing Spring Cave soil samples (9%). Little is known about this phylum, but appears to be ubiquitous in environmental soil samples. Firmicutes, specifically *Bacillus spp.*, have the ability to reduce nitrate to ammonia (Rajakumar et al.,

2008), have protease, chitinase and β-keratinase activities (Brar et al., 2009). Species from this genus have been studied as bioremediators, as antibiotic producing bacteria, and have been used in other applications. Chloroflexi (5%) include many photosynthetic bacteria, and species within this phylum have been used in the dechlorination of polychlorinated biphenyls (PCBs) and other bioremediation applications (Field et al., 2008). Acidobacteria is also a relatively new phylum and is not yet well understood, but it is prevalent in many environments, including this cave soil sample (4%). The two groups, unknown-1 and 2, each represented 6% of total sequences and clearly separate from one another (and consequently from the other eight phyla). These two groups also had no close matches (<75% homologies) in GenBank.

This study establishes a baseline for the study of microbial communities in Blowing Spring Cave and contributes to the understanding of bacterial communities in OTHERother cave systems. Further, it is possible that the loss of natural microflora, such as actinobacteria (which are know to produce antifungal compounds) could allow *Fusarium* to proliferate and infect the bat populations within the cave (Yasir et al., 2009). Loss of naturally occurring bacteria in such cave systems could also allow for the influx of invasive and/or pathogenic bacteria, which could subsequently affect yet other natural microflora and other organisms in the ecosystems. Competition or antimicrobial properties of novel microbes may remove or change the metabolic activities of the natural microflora, which could remove an important link in the native nutrient chain in these cave systems.

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DETERMINING SOURCES OF *E. COLI* POLLUTION IN DRY CREEK, ALABAMA

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ABSTRACT

Antibiotic resistance analysis was performed to determine the source, human or nonhuman, of *E. coli* contamination from Dry Creek, Alabama. The study consisted of discriminant analysis of antibiotic resistance patterns generated from *Escherichia coli* growth during exposure to eight antibiotics (ampicillin, chloramphenicol, erythromycin, neomycin, oxytetracycline, spectinomycin, streptomycin, and tetracycline). A reference database was constructed from 1152 *E. coli* isolates from the following sources: cattle, deer, and humans. The average rate of correct classification (ARCC) for human, cattle, and deer isolates was 89.9%. A total of 384 *E. coli* isolates were collected from Dry Creek during low flow, and another 384 isolates were collected during high flow. The low flow isolates were classified as cattle (10.3%), deer (52%), and human (37.6%). The high flow isolates were classified as cattle (7.5%), deer (5.5%), and human (87.1%). These results demonstrate that the antibiotic resistance phenotypes of *E. coli* within Dry Creek can vary substantially during different flows and that *E. coli* displaying antibiotic resistance phenotypes associated with human origins predominated during high flow.

INTRODUCTION

The National Water Quality Inventory (United States Environmental Protection Agency, 2004) ranks pathogens (pathogenic bacteria) as one of the most widespread pollution problems in the nation's rivers and streams. States are required by section 303(d) of the Clean Water Act and the Environmental Protection Agency Water Quality Planning and Management Regulations (40 CFR Part 130) to identify waters that fail to meet various pollution limits, including those for pathogens. In 1998, the State of Alabama Department of Environmental Management (ADEM) identified Dry Creek (Waterbody ID: AL/03150202-170_01) in Perry and Dallas Counties as not supporting its designated use as a Fish and Wildlife water due to excessive pathogen concentrations (ADEM, 1998). Dry Creek remained on the 303(d) list for 2000 and 2002 (ADEM, 2000; ADEM, 2002). ADEM recognizes fecal coliform (FC) counts as the indicator method for pathogen concentrations in Alabama (ADEM, 1998), and although FC are routinely monitored

in many public waters, tests that measure the presence/absence or concentration of FC provide no indication of the sources of contamination. Understanding the sources of FC contamination is important because the health risk to humans is greater when pathogens originate from human sources than from animal sources (ADEM, 1998).

The efficient execution of water quality maintenance, public health assessment, and environmental pollution management efforts is dependent upon the ability to differentiate between pathogens from human sources (failing septic systems and leaking sewer lines) and nonhuman sources (domestic animals, agricultural animals, and wild animals). A number of methods have been developed to differentiate pathogen sources. Phenotypic methods include the ratio of FC to streptococci (Feachem, 1975), the species-specificity of certain bacteriophages (Rusin et al., 1992), and growth patterns of FC relative to various substrates or antibiotics. Molecular methods include fatty acid profiling (Simmons, 1994), DNA fingerprinting (Devriese et al., 1993), random amplified polymorphic DNA analysis (Berg, 1994), and 16S ribosomal DNA comparison (Bernhard and Field, 2000). Phenotypic methods have proven to be less accurate, but also less expensive, so that larger data sets can be accumulated for statistical comparison.

The approach used in this study is phenotypic and is based upon the premise that antibiotic use in humans and animals can result in host-specific patterns of antibiotic resistance by the host-associated enteric bacteria (Holmberg et al.., 1984). The multiple antibiotic resistance (MAR) index was developed by testing bacterial isolates for growth inhibition in the presence of a number of single concentration antibiotics (Krumperman, 1983). The MAR index has been improved by increasing the range of antibiotic concentrations tested and by using discriminant analysis (DA) to categorize the antibiotic resistance patterns with a high degree of certainty. The improved method, antibiotic resistance analysis (ARA), has been applied to fecal pollution source-tracking with success (Wiggins, et al.., 1996; Wiggins, et al.., 1999; Hagedorn et al.., 1999; Burnes, 2003; Choi, 2003). This study compared antibiotic-resistance patterns of a subset of the FC, *Escherichia coli,* from water samples and known sources in the Dry Creek watershed. The study consisted of two components: the development of an *E. coli* reference database followed by the classification of nonpoint source *E. coli* collected from Dry Creek during low flow or high flow. Concentration series of eight different antibiotics were used to establish phenotypic patterns of *E. coli* isolates and discriminant analysis (DA) was applied to the patterns to classify the isolates as human or nonhuman.

MATERIALS AND METHODS

The Dry Creek watershed (Waterbody ID: AL/03150202-170_01; hydrologic unit 03150202) in Perry and Dallas Counties, Alabama, consists of 5,330 acres (8.3 sq. mi.) adjacent to Dry Creek, an intermittent stream 4.5 miles in length from its headwaters to its confluence with the Cahaba River. The Dry Creek watershed is a subunit of the Cahaba River basin, and land use in the Dry Creek watershed consists of cropland (1,793 acres,

2.8 sq. mi., 33.6% of total), pastureland (1,905 acres, 3.0 sq. mi., 35.7% of total), forest (1,631 acres, 2.5 sq. mi., 30.6% of total), and urban (1 acre, <0.0 sq. mi., <0.0% of total). Dry Creek was placed on the 303(d) list for high pathogen counts; example data from 1996 shows fecal coliform counts ranging from 20 – 40,000 per 100ml for a single sample and counts per 30 days were estimated to be as high as 7.74×10^{13} (USEPA, 2003).

A local E. coli antibiotic profile reference database was generated from E. coli isolates from known sources in the Dry Creek watershed. The known source E. coli were collected from either the sewage influent from the City of Marion Water Treatment Plant, or from fresh cattle or deer excreta (in plastic sample bags). Water samples were collected from an existing water quality monitoring site in the Dry Creek watershed for nonpoint source E. coli (Fig. 1). Samples were collected during low flow (<0.1 inch of rain in the preceding 72 hours) and during high flow (>0.1 inch of rain within 24 hours preceded by low flow conditions). Water samples were collected with sterile sampling bottles or pipettes and immediately aliquotted into culture media. Sewage and fecal samples were placed on ice and returned to the laboratory within 6 hours where they were suspended (1 gram/100ml) and serially diluted in FC Buffer ($3x10^{-4}$ M H_2KPO_3, $2x10^{-3}$ M $MgCl_2.6H_2O$, pH 7.2±0.2) and then processed identically to water samples.

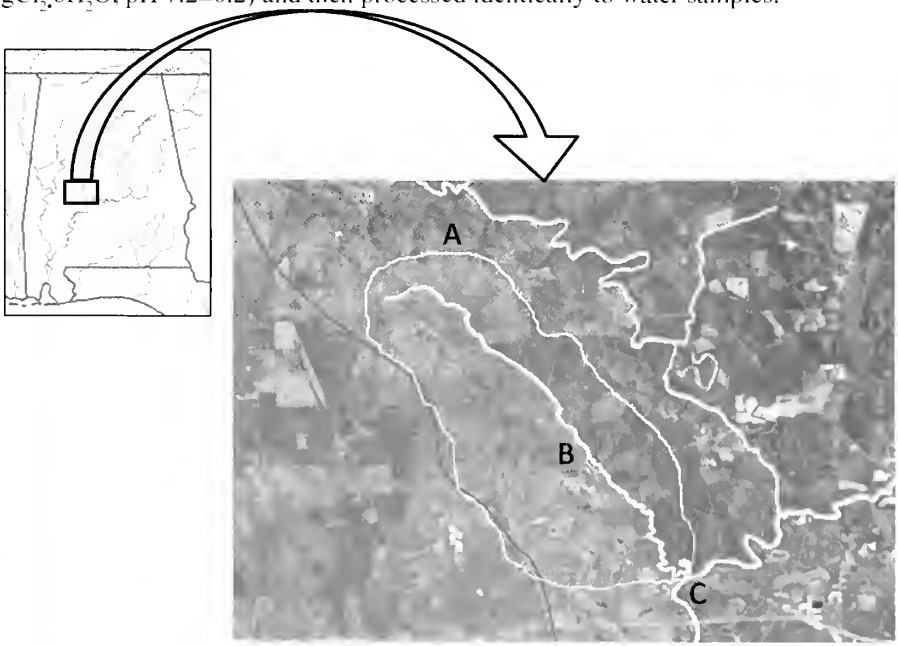

Figure 1: The location of the Dry Creek watershed in Alabama and an aerial view of the watershed. The boundary of Dry Creek watershed is the thin white line labeled "A". Dry Creek is indicated by the thicker white line labeled "B", which is adjacent to the sampling site (Latitude: 32.49036153263807; Longitude: -87.19676971435547). The confluence of Dry Creek and the Cahaba River is labeled "C". Land use is visible as lighter-shaded areas (cropland and pastureland) and darker-shaded areas (forest).

E. coli were isolated using EasyGel ColiScan plates (Micrology Labs, Inc., Goshen, Indiana) according to the manufacturer's instructions. For data consistency with ongoing monitoring efforts, this method was compared to Standard Methods 9222D (Feachem, 1975), the same method used in routine regulatory *FC* analyses, and the results of both were within 1% (data not shown). After isolation, individual blue-pigmented colonies were transferred to 96-microwell plates containing 0.2 ml of mFC broth (Difco, Detroit, MI; pH 7.4, prepared to manufacturer's specifications and used within 72 hours, stored at 4 °C) and incubated at 44.5±0.2°C for 48 hours. Wells that exhibited growth and formed a dark blue color after incubation were identified as putative *E. coli* cultures. All putative *E. coli* cultures isolates were transferred into wells of microtiter plates that contained EC broth amended with 4-methylumbelliferyl-β-d-glucuronide (MUG) (50 µg/ml). β-Glucuronidase activity, which is specific to *E. coli* in the fecal coliform group, was assessed by MUG cleavage and determined by fluorescence in the microtiter plate upon exposure to UV light (Bitton et al.., 1995). Only MUG-positive colonies were further analyzed. A reference strain, *E. coli* no. 11775 (American Type Culture Collection, Rockville, MD), was included as a positive control. A total of 384 *E. coli* isolates were collected from each sample.

A minimum inhibitory concentration (MIC) was determined for each of eight antibiotics (Sigma-Aldrich, St. Louis, MO) by assaying an initial set of *E. coli* isolates for resistance at the following final concentrations: ampicillin (12.5, 25, 50, and 100 µg/ml), chloramphenicol (1.25, 2.5, 5, and 10 µg/ml), erythromycin (12.5, 25, 50, and 100 µg/ml), neomycin (12.5, 25, 50, and 100 µg/ml), oxytetracyclin (12.5, 25, 50, and 100 µg/ml), spectinomycin dihydrochloride (1.25, 2.5, 5, and 10 µg/ml), streptomycin sulfate (12.5, 25, 50, and 100 µg/ml), and tetracycline hydrochloride (1.25, 2.5, 5, and 10 µg/ml). Sterile antibiotic solutions were added to tempered (44.5°C) mFC agar (Difco), mixed, poured into petri dishes, and allowed to cure for two days. Each set of petri dishes consisted of one plate of each concentration of each antibiotic and one control plate without antibiotics.

Cultures were transferred from 96-microwell plates to petri dishes with a sterilized 50-pin replicator fashioned from an Immunology TSP (Nalge Nunc International Corp., Naperville, IL) and incubated at 44.5±0.2°C for 24 hours. Isolates were recorded as resistant to an antibiotic if a dark blue colony was observed after incubation. Cultures that failed to develop colonies on mFC agar without antibiotics were eliminated from further analysis. Duplicates of approximately 5% of the known source isolates were performed to check for consistency. All subsequent antibiotic resistance analyses were performed at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), erythromycin (100 µg/ml), neomycin (50 µg/ml), oxytetracyclin (100 µg/ml), spectinomycin dihydrochloride (10 µg/ml), streptomycin sulfate (100 µg/ml), and tetracycline hydrochloride (10 µg/ml).

The antibiotic resistance growth patterns of each isolate were compared using discriminant function analysis (DA). DA is a multivariate technique used to separate distinct sets of observations into predefined classes and then to categorize new observations into these classes. DA generated discriminant scores that were used to classify each *E. coli* isolate according to source and determine the average rate of correct classification (ARCC)

(Wiggins, 1996). The analyses were performed with SPSS Version 16.0 (SPSS, Inc., Chicago, IL) using the linear DA option, which assumes that all groups share a common covariance matrix and that the measurement variables have a normal distribution. Prior probabilities for each group were set to equal since the groups were of the same size.

RESULTS

The inclusion of three distinct source groups allowed the generation of two discriminant functions that were applied to the antibiotic resistance profile of each *E. coli* isolate. The equations for the discriminant functions are: DF1 = Amp(0.883) + Ery(-0.008) + Oxy(0.189) + Spec(0.446) + Strep(-0.075) + Tet(0.019) + Van(-0.244) and DF2 = Amp(0.213) + Ery(-0.362) + Oxy(0.070) + Spec(-0.360) + Strep(0.138) + Tet(0.579) + Van(0.746). The discriminant function scores of known-source *E. coli* were plotted against one another using DF1 as the x value and DF2 as the y value and overlayed with the discriminant function scores of nonpoint-source *E. coli* from Dry Creek collected during low flow (Fig. 2) or high flow (Fig. 3).

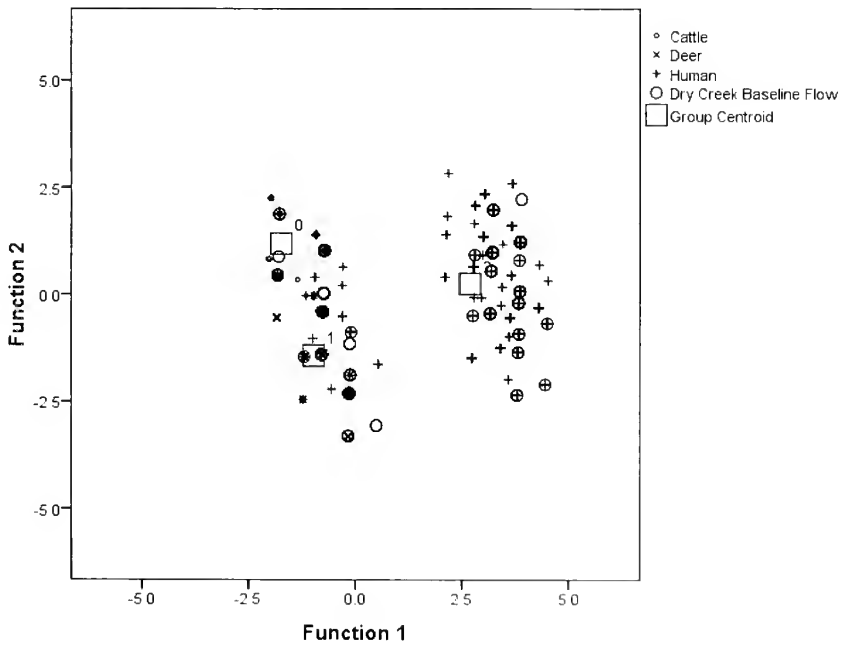

Figure 2: Discriminant analysis of *E. coli* from Dry Creek collected during baseline (low) flow. Each symbol indicates at least one *E. coli* phenotype; overlapping symbols indicate multiple *E. coli* with that phenotype; n=384 for each group. Group centroids are the three large squares labeled "0" (Deer), "1" (Cattle), or "2" (Human).

Using discriminant analysis, 95.8% of the deer *E. coli* isolates were correctly classified into the deer group (Table 1). The remaining 4.2% of the deer *E. coli* isolates were misclassified into the cattle group. Similarly, 92.2% of the cattle *E. coli* isolates were correctly classified into the cattle group and the remaining 7.8% were misclassified into the deer group. The correct classification rate for human *E. coli* isolates was 81.8%, with the remaining 18.2% misclassified as deer (5.5%) or cattle (12.8%). During low flow, 10.3% of the *E. coli* isolates from Dry Creek were classified as deer *E. coli*, 52% were classified as cattle *E. coli*, and 36.7% were classified as human *E. coli*. During high flow, 7.5% of the *E. coli* isolates from Dry Creek were classified as deer *E. coli*, 5.5% were classified as cattle *E. coli*, and 87.1% were classified as human *E. coli*. Each group consisted of 384 isolates, except the *E. coli* from Dry Creek collected during high flow, which consisted of 348 isolates. The Chi square value for the difference in numbers of human *E. coli* from low and high flow was less than $p<0.005$, indicating a 99.5% chance that the observations were non-random.

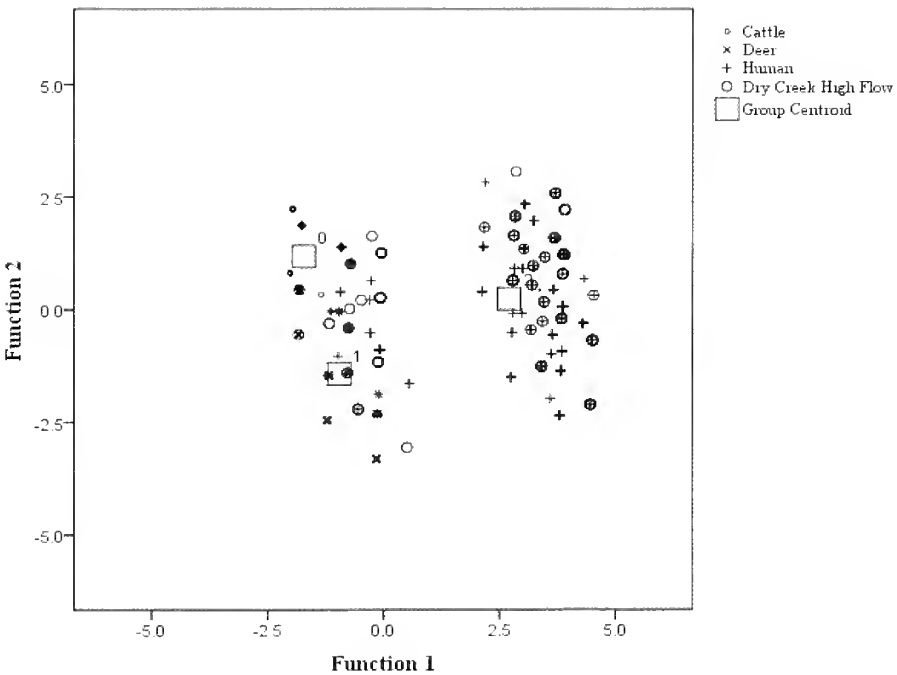

Figure 3: Discriminant analysis of *E. coli* from Dry Creek collected during high flow. Each symbol indicates at least one *E. coli* phenotype; overlapping symbols indicate multiple *E. coli* with that phenotype; n=384 for each group, except Dry Creek (n=348). Group centroids are the three large squares labeled "0" (Deer), "1" (Cattle), or "2" (Human).

Table 1. Enumeration of classified *E. coli* from reference sources and Dry Creek collected during low flow and high flow. Percentages are in parentheses.

	Classification			
Source	**Deer**	**Cattle**	**Human**	**Total**
Deer	**368 (95.8)**	**16 (4.2)**	**0**	**384**
Cattle	**30 (7.8)**	**354 (92.2)**	**0**	**384**
Human	**21 (5.5)**	**49 (12.8)**	**314 (81.8)**	**384**
Dry Creek - Low Flow	**36 (10.3)**	**181 (52.0)**	**131 (37.6)**	**384**
Dry Creek - High Flow	**26 (7.5)**	**19 (5.5)**	**303 (87.1)**	**348**

DISCUSSION

While *E. coli* is the indicator of pathogens associated with fecal contamination for regulatory agencies, there is considerable evidence to suggest that the majority of these strains do not cause severe human diseases (Ishii et al., 2007). In addition, the persistence of *E. coli* in streams (Sherer et al., 1992) and the growth of *E. coli* in nonhost environments has been reported previously (Solo-Gabriele et al., 2000; Hardina and Fujioka, 1991), suggesting that the detection of source-type *E. coli* may not strongly correlate to recent contamination. The natural genetic diversity of *E. coli* in environmental samples has been demonstrated to be enormous (McLellan, 2004), and it has been demonstrated that bacteria in the soil can acquire resistance to tetracycline from environmental exposure, possibly creating a reservoir of resistance factors generated outside host animals (Rysz and Alvarez, 2004). This finding suggests that the collection of nonpoint-source samples from a watershed may be a more accurate measure of exposure to resistance factors from farm runoff rather than direct measurement of source loading in watersheds.

Although no one statistical method has been shown to best categorize microbes in source tracking studies, discriminant function analysis is one of the most commonly used techniques (Ritter et al., 2003). In order to make discriminant function analysis as robust as possible, multiple isolates with the same antibiotic resistance profile from each source were deleted, a practice known as decloning, to avoid artificial inflation of correct classification rates (Stoeckel and Harwood, 2007). In addition, temporal changes in *E. coli* populations from waterfowl species have been demonstrated; therefore, no waterfowl sources were included in this study (Hansen et al., 2009). Various studies have demonstrated that known-source libraries with fewer than 2,000 isolates have the highest accuracy when the isolates are collected within the watershed, rather than applying a database from another area (Hartel et al., 2002; Wiggins et al., 2003). Therefore, all of the reference isolates for this study were collected within the watershed except for the human-source *E. coli*, which were collected from the closest wastewater treatment facility. Studies in which over 2,400 *E. coli* were analyzed indicate that even larger sample sets may

be needed to sufficiently represent the extremely large number of environmental microbes (Johnson et al., 2004); however, smaller sample sets such as the one used in this study are sufficient when the classifications are accordingly broad (i.e., human versus nonhuman).

No visible source of human contamination was observed in Dry Creek, although a human population of approximately 84 individuals uses on-site wastewater treatment systems in the watershed (United States Environmental Protection Agency, 2003). Cattle and deer excreta are readily observable in the watershed, and these were sampled for reference or known-source nonhuman *E. coli*. A total of 768 *E. coli* were pooled for the reference nonhuman group, and none demonstrated a human-type antibiotic resistance pattern. Although there were significant ($P < 0.05$) differences between the means of all of the known-source *E. coli* discriminant function scores, the discriminant function scores of deer and cattle *E. coli* overlapped one another and were grouped around centroids 0 and 1, respectively, from coordinates x=-2.5 – 0.5 and y=-3.5 – 2.5 (Fig. 2 and 3). Additionally, the deer and cattle scores overlapped a subset of the human scores at coordinates x=-2.5 – 0.5 and y=-2.4 – 2.0. However, no deer or cattle scores were found with an x value greater than 1.0, suggesting that x=1.0 is a threshold value for differentiating human from non-human *E. coli* in Dry Creek.

The relative similarity of non-human scores for discriminant function 1 (Fig. 2 and 3) found in this study is consistent with previous studies (Burnes, 2003; Hagedorn et al., 1999) and is the basis for structuring this study comparison as human versus non-human. Although the non-human *E. coli* isolates were correctly classified at high rates (deer, 95.8%; cattle, 92.2%), the remaining non-human *E. coli* isolates were misclassified as the alternate non-human group, not as human. This means that no *E. coli* from deer or cattle were mistaken for human *E. coli*. An independent set of human *E. coli* scores was grouped around centroid 2 from coordinates x=2.4 – 5.0 and y=-2.5 – 2.5. The correct classification rate for these human *E. coli* isolates was 81.8%, with the remaining 18.2% misclassified as deer (5.5%) or cattle (12.8%). Although 18.2% of human *E. coli* were mistaken for *E. coli* from deer and cattle, these results are still statistically significant in a two-group system (human or non-human) where any classification rate above 50% is better than random.

Nonpoint-source *E. coli*, collected from Dry Creek during either low or high flow, had scores that were distributed throughout the ranges of all three reference groups. During low flow, 10.3% of the *E. coli* isolates from Dry Creek were classified as deer *E. coli*, 52% were classified as cattle *E. coli*, and 36.7% were classified as human *E. coli*. During high flow, 7.5% of the *E. coli* isolates from Dry Creek were classified as deer *E. coli*, 5.5% were classified as cattle *E. coli*, and 87.1% were classified as human *E. coli*. Each group consisted of 384 isolates except the *E. coli* from Dry Creek collected during high flow, which consisted of 348 isolates (Table 1). The concomitant and significant increase in human-classified *E. coli* with the decrease in cow-classified *E. coli* suggests that human fecal contamination washes into Dry Creek during high flow, and the large wash volume dilutes cow fecal contamination. The source of human contamination must be on the surface, otherwise the baseline contamination levels wold be higher. Possible sources of human contamination include improperly functioning on-site wastewater treatment systems, such as leaking sanitary plumbing or faulty septic tanks, which could

release untreated sewage into surface waters. Although, further studies are necessary to positively identify putative human sources of fecal contamination, the limited number of human inputs may make the identification of sources of contamination possible through field surveys of the Dry Creek watershed.

This study provides the first evidence from Dry Creek that total fecal coliform and *E. coli* counts consist of populations of cells that originate from different host organisms. *E. coli* displaying antibiotic resistance phenotypes associated with human origins predominates during high flow in Dry Creek, and *E.coli* from deer and cattle are significantly differentiable from those of humans. These findings will aid in determining if the *E. coli* present in Dry Creek constitute a threat to human health. In addition, the ability to further subdivide the *E. coli* loads into source groups, such as deer or cattle, will help in the development of management and TMDL plans for Dry Creek. Ongoing studies will apply these findings to the investigation of temporal changes in *E. coli* populations in Dry Creek.

ACKNOWLEDGEMENTS

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ENHANCEMENT OF CALLUS INDUCTION AND CUCURBITACIN CONTENT IN *CITRULLUS COLOCYNTHIS* L. (SCHRAD) USING PLANT GROWTH REGULATORS

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ABSTRACT

The objectives of this study were to investigate the effect of growth regulators and different explant type in callus induction and to increase the yield of cucurbitacin in leaf, stem and root explants of *Citrullus colocynthis*. The specimens were 15 days old and were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 2, 4- dichlorophenoxy acetic acid (2, 4-D), Kinetin (kin), Benzyl adenine (BA) and α-naphthalene acetic acid (NAA) The different concentrations of 2, 4-D + kin and BA + NAA as well as different explant organ types increased the callus fresh weight and dry weight. The callus cultures derived from stem explants grown on BA + NAA were proved to be an appropriate protocol for callus induction, while 2,4-D failed to stimulate callus growth in the same manner. Different callus explants and the *in vitro* raised seedling leaf, stem and root were harvested and subjected to extraction of active principle compounds. The results revealed that stem-derived callus cultured on 2,4-D (2 mg/l) + kin (4 mg/l) produced the highest total cucurbitacins content with values reaching 10.89% compared to the control seedling stem, leaf and root which produced (4.95, 4.97 and 5.12%), respectively. The HPLC analysis of cucurbitacin-E showed distinct changes in the different cultures initiated from various explants.

INTRODUCTION

Many desert plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavors, fragrance ingredients and food additives. *Citrullus colocynthis* L. (Schrad) is commonly known as Sherry or Handal and is widely distributed in the Egyptian deserts. The plant fruits are used in folk medicine by nomads and people in rural areas as a purgative, anthelmintic, carminative, anti-rheumatic, anti-diabetic and antipyretic (Maatooq et al., 1997; Nmila et al., 2000; Hegazy, 2007). Phytochemical analysis of the fruits and leaves of the plant demonstrated the presence of flavonoids, caffeic acid derivatives and terpenoids such as cucurbitacins (Hatam et al., 1989; Seger et al., 2005).

The cucurbitacins (highly oxygenated tetracyclic triterpens) were investigated for their cytotoxic, anti-inflammatory, hepatoprotective and cardiovascular effects (Liu et al., 2008). Additionally, several studies indicated that different forms of cucurbitacins inhibit the proliferation of cancer cells and induce cell apoptosis (Jing and Tweardy, 2005; Sun et al., 2005).

In order to protect the medicinal plant resources, cell culture has been used as an alternative source of active constituents of medicinal plants with a view of enhancing secondary metabolites. The potential benefits of stimulating the formation of desirable medicinal compounds from plants, especially by using biotechnological approaches such as plant tissue culture has been recognized (Ramachandra Rao and Ravishankar, 2002). For example, ginsenoside from Panax *gingseng*, rosmarinic acid from *Coleus blumei*, shikonin from *Lithospermum erythrolizon*, diosgenin from *Dioscorea*, ubiquinone-10 from *Nicotiana tabacum*, berberin from *Coptis japonica*, and podophyllotoxin from *Juniperus chinensis* all accumulated at much higher levels in cultured cells than in intact plants (Misawa et al., 1985; Smith, 2002; Premjet et al., 2002). Several attempts were made in order to obtain a high yield of secondary metabolites by applying exogenous phytohormones or chemical treatments in the growth media (Satdive et al., 2007; Shinde et al., 2009). Establishment of an efficient callus protocol is a prerequisite for harnessing the advantages of cell and tissue culture. Induction of callus biomass and physical disorganization of cultured cells is thought to be a result of the breakdown of intercellular physical and chemical communication (Lindsey and Jones, 1992).

Plant growth regulators such as auxins (2,4-D and NAA) and cytokinins (BA and kin) have different effects on plant growth and are often crucial factors in secondary metabolite accumulation (DiCosmo and Towers, 1984). On the other hand, NAA stimulates water absorption, enhances cell wall plasticity and initiates callus formation (Aki, 2005). The quality and quantity of plant growth regulators initially presented in the media or administered during the course of *in vitro* culture proved to have a significant effect on the metabolism of secondary metabolites (Zia et al., 2007). Since the production of secondary metabolites in plant cell culture is a function of both cell multiplication and division, growth regulators are bound to play a major role in determining the production potential of a given culture (Staba, 1980). The effect of auxin type on secondary metabolite synthesis was studied in the tissue culture of *Ecballium elaterium*. Higher yields of cucurbitacin B (1.126%) were obtained from stem node callus cultures in the presence of BA (1 mg/l) and NAA (0.1 mg/l) compared with the yield obtained from raw plant material (0.01%) as stated by Toker et al. (2003).

The present study was carried out to develop an efficient protocol for callus induction, total cucurbitacins and cucurbitacin-E accumulation under the effect of different growth regulators and explant types of *Citrullus colocynthis* (Handel).

MATERIALS AND METHODS

Standards and Reagents

Cucurbitacin-E, purity (HPLC)>98% was obtained from Carl Roth GmbH+Co. KG. (Karlsruhe, Germany); MS media, 2,4- dichlorophenoxy acetic acid (2,4-D), Kinetin (kin), Benzyl adenine (BA) and α-naphthalene acetic acid (NAA) were obtained from Sigma Chemical Co. (St. Louis, Mo. USA); and phosphomolybdic acid was obtained from BDH, Dorset, UK. The HPLC-grade organic solvents methanol and acetonitrile were obtained from Merck. All other chemicals were of analytical-grade purity and available from the Centre of Excellence for Advanced Sciences, National Research Centre, Dokki, Egypt.

Plant Materials

Mature seeds of Handal were collected from naturally growing plant populations in Wadi Soule, Sinai, Egypt. The collected seed materials were botanically authenticated (collection No. 655) by the Herbarium of the Botany Dept., Faculty of Science, Cairo University. The collected seed material was kept in dark bottles and preserved under normal lab conditions until used.

Culture Media

MS-media (Murashige and Skoog, 1962) was used as the base medium. The medium was solidified with 0.7% agar and pH adjusted to 5.8 before autoclaving. The prepared media were poured into sterilized 200 ml glass jars. Each jar contained 50 ml solidified medium; the media were then autoclaved at 121°C under pressure 1.2 Kg/cm^2 for 20 min.

Seed Germination and Explants

Seeds of *C. colocynthis* were manually decoated and then transferred to the laminar air flow cabinet. The seeds were kept in 70% ethanol for 30 sec followed by rinsing three times in sterile distilled water. The seeds were then immersed in a continuously stirred 50% (v/v) commercial Clorox solution (5.25% NaOCl) for 30 min and finally washed several times with sterile distilled water to remove any traces of Clorox. Sterilized seeds were cultured (10 seeds/jar) into 200 ml glass jars containing 50 ml of solidified basal MS-medium supplemented with 3% sucrose and 0.7% agar without growth regulators. The jars containing the seeds were incubated for seed germination in the dark at 28±2 °C for five days. Jars with germinated seeds were then transferred to the growth chamber at 25±2 °C and 16/8 h light/dark regime at light intensity 2000 Lux produced from cool white fluorescent lamps. After 15 days, the *in vitro* raised seedlings were used as the plant material source for callus induction.

Callus Induction

Three explants (leaf, stem and root) were excised from *in vitro* raised seedlings

and cultured in 200 ml jars containing 50 ml MS-medium. To assess the effect of auxin in combination with cytokinin on callus induction, eight different medium formulations were used. All the formulations consisted of MS -medium supplemented with different concentrations of 2, 4-D and Kin as well as BA and NAA (Table 1). The cultures were incubated for callus induction in the growth chamber for three weeks. After 21 days, callus tissues characteristics were determined. After obtaining the fresh weights, the samples were then air dried at 40°C till they reached a constant dry weight.

Table 1. Murashige and Skoog media supplemented with different concentration of growth regulators for callus induction of *Citrullus colocynthis*.

No. of media	Media code	Growth regulator concentration (mg/l)
1	MD1	MS + 2.0 2,4-D + 1.0 kin*
2	MD2	MS + 1.0 2,4-D + 1.0 kin
3	MD3	MS + 6.0 2,4-D + 2.0 kin
4	MD4	MS + 2.0 2,4-D + 4.0 kin
5	MB1	MS + 0.0 BA + 5.0 NAA
6	MB2	MS + 0.01 BA + 1.0 NAA
7	MB3	MS + 0.1 BA + 5.0 NAA
8	MB4	MS + 1.0 BA + 0.1 NAA

* Kinetin (kin), Benzyl Adenine(BA), α-Naphthalene Acetic Acid (NAA)

Determination of Total Cucurbitacins

Different callus explants and the *in vitro* raised seedling stem, leaf and root were harvested and subjected to extraction of cucurbitacins. Exactly 100 mg of each air-dried callus culture and *in vitro* seedlings stem, leaf and root organ (used as control) were ground to fine powder, followed by extraction with absolute ethanol. Each sample was extracted with 5 ml absolute ethanol for 2 hr; then the extract was filtered using Whatman No. 4 filter paper. The filtrates were dried under vacuum at 40°C using a rotary evaporator (Buchi, Switzerland) for 30 mints. The extraction was repeated twice, and the resulting residue was re-dissolved with ethanol. An equal volume of each extract and 2% solution of phosphomolybdic acid in absolute ethanol were mixed at room temperature, and the absorbance was measured by a spectrophotometer (UNICAM UV 300) at 492 nm after 5 min (Attard and Scicluna-Spiteri, 2001). Standard stock solution of cucurbitacin-E was prepared in the range between 0.5 to 5.00 mg/ml. The results were expressed as percentage (w/w) based on dry weight.

Determination of Cucurbitacin-E

Cucurbitacin-E was determined in the extract of both derived calli and *in vitro* seedling organs by High Performance Liquid Chromatography (HPLC) according to Toker et al. (2003). Dried tissues were ground to fine powder in a mortar; 50 mg of this powder was

extracted with 5 ml chloroform for 2 hr, and then the extract was filtered using Whatman No. 4 filter paper. The chloroform extract was evaporated at 45 °C till dryness. HPLC was performed using an Agilent 1100 series (Waldbronn, Germany) equipped withquaternary pump (G 1311A), degasser (G 1329A) and auto sampler (G 1329A). Dry samples were re-dissolved in HPLC-grade methanol and filtered through a Millipore filter membrane (0.45 µm pore size) directly into auto sampler vials. Optimal separation of cucurbitacin-E was obtained with isocratic mobile phase: acetonitrile / water (70:30). Flow rate was adjusted to 1ml/min and the column was equilibrated for 10 min with the mobile phase before each run. Detection was achieved by variable wave length detector (G 1314A) at wave length 229 nm. Cucurbitacin-E was identified through its retention time by comparison with the cucurbitacin-E reference standard, and quantified on the basis of the integrated peak area. The results were expressed as percentage (w/w) based on dry weight.

Statistical Analysis

The experiment was conducted as Completely Randomized Design (CRD) with five replications. The results were reported as mean ± standard deviation (SD). The significance of differences among different combinations of growth regulators and explant types was analyzed by one-way ANOVA using SPSS 10.0 Windows package. The least significant difference (LSD) at $P \leq 0.05$ level was used for mean separations for the variables with significant F values, at 5 % level of probability.

RESULTS AND DISCUSSION

Induction of Callus Cultures and Biomass Accumulation

A reliable protocol for the *in vitro* callus induction of Handal was optimized. For callus induction, eight different media formulation were used (Table 1). Callus tissues began to appear on wounded explants within one week of culturing. Callus induction efficiency expressed as fresh and dry weight differed significantly ($P \leq 0.05$) among the different concentrations of growth regulators and explant types. Efficiency of the stem explants was significantly higher than that of leaf and root explants for all media. The highest yield of callus biomass (581 mg fw/inoculum and 31 mg dw/inoculum) was obtained from stem explants raised on MD4 media (Table 2). This callus was solid in texture and pale green in color. The maximum fresh biomass in the leaf explants reached 355 mg fw/inoculum and 29 mg dw/inoculum in MB3 media (Table 3); this callus was solid in texture and white in color. The root explants produced the highest fresh biomass (173 mg fw/inoculum and 11 mg dw/inoculum) when grown on MB2 media (Table 3).

Table 2. Callus fresh and dry weight (mg/inoculum) of *Citrullus colocynthis* raised on Murashige and Skoog media supplemented by different combinations of 2,4-D and Kinetin.

Media code	Fresh and dry weight (±SD) mg/inoculum					
	Explant types					
	Leaves		Stems		Roots	
	F.W	D.W	F.W	D.W	F.W	D.W
MD1	194 ± 6	17±0.2	362 ± 9	24 ± 0.1	41 ± 9	0.7± 0.1
MD2	134 ± 2	13 ± 1	323 ± 2	27 ±0.1	78 ± 7	0.8 ± 0.3
MD3	91± 8	14 ± 1	299 ± 5	23 ± 0.2	56 ± 8	0.8± 0.2
MD4	107 ± 10	16 ± 2	581 ± 9	31 ± 1	168± 16	1.5 ± 0.7
LSD at 0.05	6.824	2.833	6.799	1.136	8.156	0.525

Table 3 Callus fresh and dry weight (mg/inoculum) of *Citrullus colocynthis* raised on medium supplemented by different combinations of Benzyl Adenine and α-Naphthalene Acetic Acid.

Media code	Fresh and dry weight (±SD) mg/inoculum					
	Explant types					
	Leaves		Stems		Roots	
	F.W	D.W	F.W	D.W	F.W	D.W
MB1	251± 19	16 ± 1	157 ± 6	21± 4	77 ± 6	5 ± 0.5
MB2	74 ± 9	12 ± 2	179 ± 6	25 ± 1	173 ± 10	11 ± 0.8
MB3	355 ± 9	29 ± 0.9	257 ± 5	27 ± 1	82 ± 5	4 ± 0.9
MB4	151 ± 10	17± 1	424 ± 4	35 ± 0.6	69 ± 9	4± 0.7
LSD at 0.05	8.728	1.037	12.248	3.153	14.636	0.341

Table 4. Callus characteristics of *Citrullus colocynthis* raised on Murashige and Skoog media supplemented by different combinations of 2,4-D and Kinetin.

Media code	Callus characteristic					
	Explant types					
	Leaves		Stems		Roots	
	Texture	Colour	Texture	Colour	Texture	Colour
MD1	solid	light brown	solid	pale green	solid	brown
MD2	solid	light brown	solid	pale yellow	solid	brown
MD3	solid	light brown	solid	pale green	solid	brown
MD4	solid	light brown	solid	pale green	solid	brown

This callus was white in color and less solid in texture. The callus characteristics on MS-medium supplemented by different combinations of growth regulators were summarized (Tables 4&5).

The effect of auxins and cytokinins and their combinations on callus fresh and dry weight indicated that kin concentration outweighs the 2,4-D in the medium. This combination produced significant effects on the callus induction in both stem and root explants. The higher concentration of 2, 4-D over kin in the medium produced significant callus biomass in the leaf explants. These results are in agreement with the findings of Halaweish and Tallamy, (1998). They confirmed that MS-medium supplemented by 2 mg/l 2,4-D in combination with 1 mg/l kin produced the highest biomass of callus tissues when induced from the rootless seedlings explants of *Cucurbita andreana*. The optimum medium for callus induction of *Eremochloa ophiuroides* (Munro) was MS media supplemented with 2,4-D at 1.0 mg/l (Yuan et al., 2009).

Table 5. Callus characteristics of *Citrullus colocynthis* raised on Murashige and Skoog media supplemented by different combinations of Benzyl Adenine and α-Naphthalene Acetic Acid.

Media code	Callus characteristic					
	Explant types					
	Leaves		Stems		Roots	
	Texture	Colour	Texture	Colour	Texture	Colour
MB1	solid	light brown	solid	pale yellow	less solid	white
MB2	solid	light brown	solid	pale yellow	less solid	white
MB3	solid	white	solid	Fluorescent green	less solid	white
MB4	solid	white	less solid	white	less solid	white

In the present study, increasing the NAA concentration in the medium amplified the callus induction in the leaf and root explants, whereas the higher ratio of BA in the medium had a positive effect on the callus induction in the stem explants. The media supplemented with BA alone or in combination with NAA were better for callus induction from seedling explants of *Ecballium elaterium* (Attard and Scicluna-Spiteri, 2001). Moreover, the highest

callus growth of *Solanum nigrum* in terms of fresh and dry weight (1818.20±63.30 and 1317.18±42.83 mg, respectively) was observed in MS medium fortified with 3 mg/l IAA and 0.5 mg/l BAP, respectively (Yogananth et al., 2009).

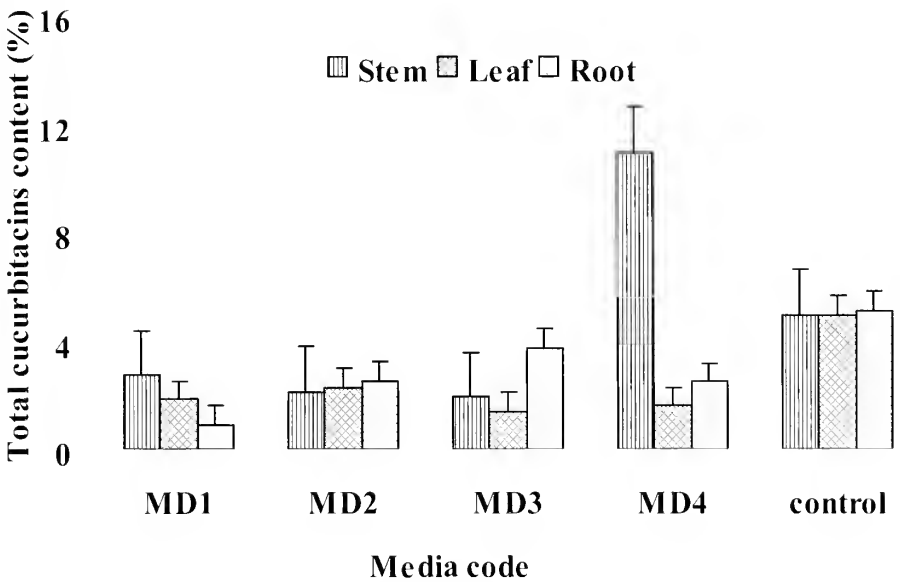

Figure 1. Total cucurbitacin content of C. colocynthis callus cultures raised on Murashige and Skoog media supplemented by different combinations of 2, 4-D and Kinetin.

Total Cucurbitacins Formation

Tissues obtained from stem, leaf and root organs of *in vitro* raised *C. colocynthis* seedlings and their derived callus cultures were analyzed for their total cucurbitacins content. The total cucurbitacin content in the ethanolic extracts differed significantly ($P<0.05$) depending on the explant type and the different concentration of growth regulators. Data presented in Figures 1and 2 shows that the highest content of total cucurbitacins (10.89%) was produced in the stem- derived calli cultured on MD4 media. This content was higher than that in the control seedling stem, leaf and root. The *in vitro* production of total cucurbitacins was increased from 0.02±0.003 mg/g, fresh weight with 2 mg l 2, 4-D and 1 mg/l kin to 0.04±0.004 mg/g, fresh weight with 6 mg/l 2, 4-D and 2 mg l kin in callus cultures of *Cucurbita andreana* as reported previously (Halaweish and Tallamy, 1998).

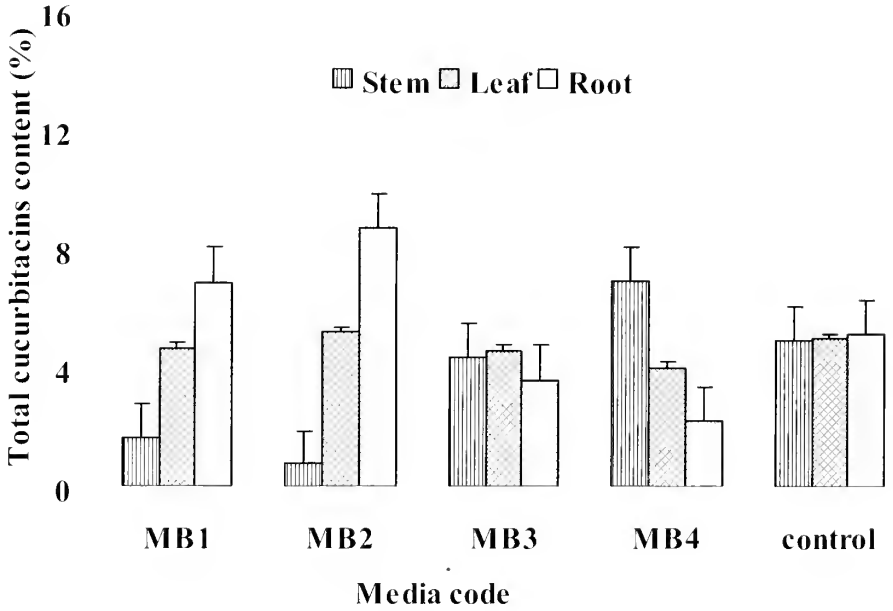

Figure 2. Total cucurbitacin content of *C. colocynthis* callus cultures raised on Murashige and Skoog media supplemented by different combinations of Benzyl Adenine and α-Naphthalene Acetic Acid.

The effect of various concentrations of BA and NAA treatments on total cucurbitacins production presented in Figure 2 shows that the total cucurbitaeins content attained the highest value (8.68%) in the root-derived calli raised on MB2 media. The higher concentration of NAA over BA in the medium (MB2) caused significant total cucurbitacins production in the callus cultures of leaf and root explants (5.19 and 8.68%) compared to the yields obtained from *in vitro* seedling leaf and root (used as control) 4.97 and 5.12%, respectively. The high concentration of BA in the medium (MB4) had a greater effect on the production of total cucurbitacins in the stem explants (6.93%) than in the *in vitro* seedling stem used as control (4.95 %).

These results are in agreement with those reported by Attard and Scicluna-Spiteri, (2001). They demonstrated that media containing 5 mg/l NAA optimized the total cucurbitacin content (3.064%) in the undifferentiated callus cultures of *Ecballium elaterium*. Additionally, increasing the level of NAA on the MS media supplemented with 2 mg/l NAA and 0.5 mg/l BA produeed the highest aceumulation of Phyllanthusol A (20.23 mg/ g, dry weight) in eallus eultures of *Phyllanthus acidus* Skeels (Duangporn and Siripong, 2009). It is evident from the data of Figures 1&2 that 2, 4-D in combination with kin have a greater effect on the *in vitro* production of total cucurbitacins than BA in eombination with NAA. Secondary metabolites are enhanced by over-expression of the enzymes that often link

the secondary metabolic pathways to the primary metabolic pathways *viz*, cucurbitadienol synthase is the first pathway-specific enzyme in cucurbitacin biosynthesis (Balliano et al., 1983). The enhancement of total cucurbitacins in *C. colocynthis* callus cultures may be attributed to the positive effect of the plant growth regulators on cucurbitadienol synthase activity.

Content of Cucurbitacin-E

HPLC analysis was used to detect cucurbitacin-E concentration in the callus cultures and *in vitro* raised seedling leaf, stem and root organs. Generally, the accumulation of cucurbitacins-E was higher under 2, 4-D + kin treatments than BA+NAA treatments (Figure 3). The high concentration of 2,4-D over kin in the media (MD3) showed significant superiority for cucurbitacin-E production (0.16%) in the root explants. This level is higher than those estimated from the *in vitro* raised seedling stem, leaf and root, which were estimated at 0.023, 0.083, and 0.013%, respectively. The high concentration of kin in the media (MD4) significantly increased cucurbitacin-E concentration (0.026%) in the stem explants whereas the equal concentrations of 2,4-D and kin in the media (MD2) showed significant superiority for cucurbitacin-E production (0.065%) in the leaf explants.

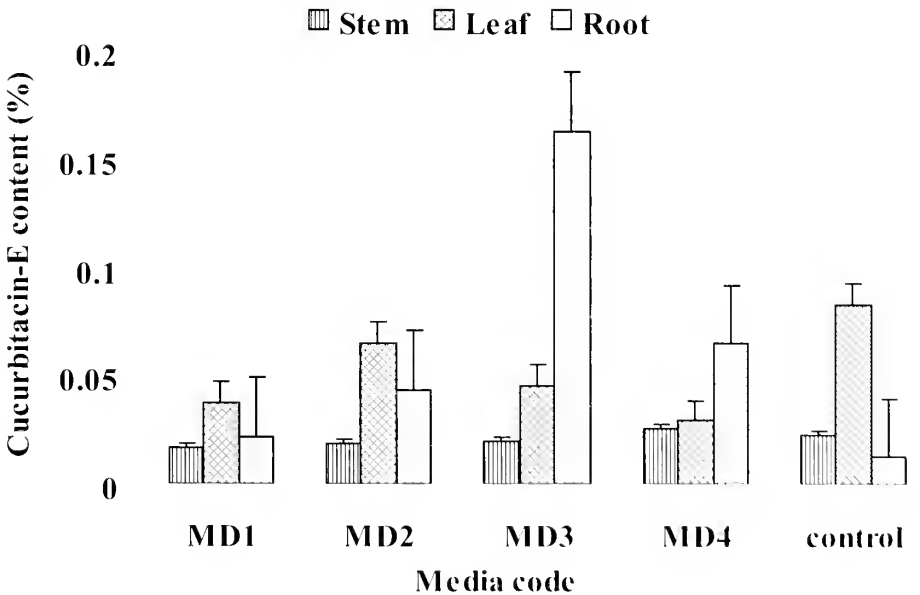

Figure 3. Cucurbitacin-E content of *C. colocynthis* callus cultures raised on Murashige and Skoog media supplemented by different combinations of 2,4-D and Kinetin.

Data presented in Figure 4 demonstrate the effects of different concentrations of BA and NAA on cucurbitacin-E production. Among single growth regulators, increasing the

concentration of NAA in the media (MB3) caused significant increases in cucurbitacin-E in the leaf and root explants with values 0.0337 and 0.0886%, respectively, over all the other studied growth regulator treatments. The higher concentration of BA in the medium (MB4) exerted a positive effect on the accumulation of cucurbitacin-E 0.077% in the stem explants.

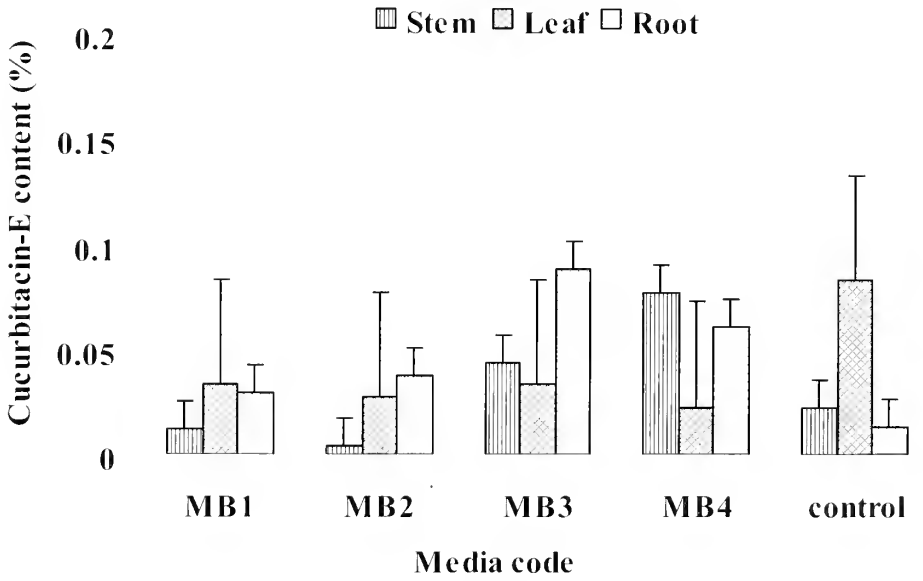

Figure 4. Cucurbitacin-E content of *C. colocynthis* callus cultures raised on Murashige and Skoog media supplemented by different combinations of Benzyl Adenine and α-Naphthalene Acetic Acid.

These results are in agreement with those of Attard and Scicluna-Spiteri, (2001), who demonstrated that MS media containing 5 mg/l NAA produced cucurbitacin-E content of 2.970% in undifferentiated callus cultures of *Ecballium elaterium*. Additionally, Nikolaeva et al., (2009) reported that the presence of NAA in the nutrient medium elevated the biosynthetic ability of callus cultures of tea plant (*Camellia sinensis* L.) and especially stimulated the accumulation of total soluble phenolics. Higher yields of cucurbitacin B (1.126%) were obtained from stem nodes callus cultures of *Ecballium elaterium* in the presence of 1 mg/l BA and 0.1 mg/l NAA compared to the yield obtained from raw plant material 0.01% (Toker et al., 2003). In conclusion, the effectiveness of *Citrullus colocynthis* callus culture induction, total cucurbitacins and cucurbitacin-E depends on the type and concentration of growth regulator as well as the explant source organ. In a majority of cases, the BA in combination with NAA was the best treatment for callus induction. The stem explants demonstrated better callus induction than leaf and root. Also *C. colocynthis* proved to synthesize total cucurbitacins and cucurbitacin-E in undifferentiated callus. Using of 2, 4-D in combination with kin was found to be the best treatment for cucurbitacins production. The accumulations of total cucurbitacins and cucurbitacin-E in callus tissues

were higher than those obtained from the *in vitro* raised seedlings. This study suggested that *in vitro* secondary metabolites production by *C. colocynthis* callus cultures could be considered an appropriate alternative method to whole plant extraction.

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THE GENUS *INDIGOFERA* (FABACEAE) IN ALABAMA

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ABSTRACT

The goals of this project were to determine which taxa of *Indigofera* occur in Alabama and to report the distribution of each. *Indigofera* (Fabaceae), commonly known as indigo, consists of four species in Alabama. The most common species is *I. caroliniana* Miller. The less common species are *I. hirsuta* Linnaeus, *I. tinctoria* Linnaeus and *I. miniata* Ortega var. *leptosepala* (Nuttall *ex* Torrey & A. Gray) B. L. Turner. The dichotomous key and descriptions were generated based on morphological features of vegetative and reproductive structures reported in the literature and of more than 200 specimens studied. County-level distribution maps were compiled entirely from herbaria vouchers.

INTRODUCTION

Indigofera, commonly known as indigo, is a member of the legume family Fabaceae (Leguminosae), subfamily Papilionoideae, tribe Indigoferinae (Schrire, 1995). The Indigoferinae, primarily a tribe of the Old World tropics, is recognized as being derived from a woody Tephrosieae (=Millettieae) (Pohill, 1981). Findings by Doyle *et al.* (1997), using *rbcL* sequence data, support this classification.

Indigofera consists of approximately 700 species worldwide and occurs on all major land masses, but is most abundant in Africa and Asia (Isely, 1990). In the United States, *Indigofera* consists of both native and introduced taxa (Isely, 1990). Fifteen species and four infraspecific taxa of have been reported from the United States. Of these, 12 species have been reported from the southeastern United States (USDA, NRCS, 2010).

Indigofera tinctoria Linnaeus was introduced into the United States in the seventeenth century and cultivated as a source of indigo dye, which was an important commodity of commerce until it was replaced by synthetic dyes in the late nineteenth century (Isely, 1990).

MATERIALS AND METHODS

Distribution maps are based on more than 200 plant specimens deposited in the herbaria of Troy University (TROY), J. D. Freeman (AUA), The University of Alabama

(UNA), The University of South Alabama (USAM), Jacksonville State University (JSU), University of North Alabama (UNAF), and Vanderbilt University (VDB), which is housed at the Botanical Research Institute of Texas (BRIT) in Fort Worth.

Herbarium specimens were initially divided into groups based on overall morphological similarity and the species concept established by Isely (1990). The dichotomous key is a modification of Isely (1990) and Weakley (2007). Descriptions for each taxon are based on Isely (1990). For *Indigofera caroliniana* Miller and *I. hirsuta* Linnaeus, morphological measurements were taken from selected specimens from throughout the geographical range in Alabama and incorporated into the descriptions if they differed from Isely (1990). Since both *I. tinctoria* Linnaeus and *I. miniata* Ortega var. *leptosepala* (Nuttall *ex* Torrey & A. Gray) B. L. Turner are known from single collections in the state, morphological measurements are entirely from Isely (1990), which allows for the range of variation throughout the southeastern United States. Illustrations are by the first author. The lists of specimens examined are limited to one record from each county.

RESULTS

Four species of *Indigofera* occur in Alabama. The most common species is *I. caroliniana* Miller (14 counties). The less common species are *I. hirsuta* Linnaeus (three counties), *I. tinctoria* Linnaeus (one county) and *I. miniata* Ortega var. *leptosepala* (Nuttall *ex* Torrey & A. Gray) B. L. Turner (one county).

TAXONOMIC TREATMENT OF *INDIGOFERA*

Indigofera Linnaeus, Sp. Pl. 751. 1753.
Anil (Ludwig) Miller, Gard. Dict. abr. ed. 4. 1754.
Bremontiera de Candolle, Mem. Legum. part 5. Paris. 249-312. 1825.
Hemispodon Endlicher, Flora. 15: 385. 1832.
Eilemanthus Hochstetter, Flora. 29: 593. 1846.
Amecarpus Bentham, Lindl. Veg. Kingd. 554. 1847.
Indigastrum Jaubert & Spach, Illust. Pl. Orient. pl. 492. 1857.
Anila Kuntze, Rev. Gen. 160. 1891.
Vaughania S. Moore, J. Bot. 58: 188. 1920.

Herbs or shrubs. Stems prostrate, sprawling, ascending or erect, strigose, pilose or hirsute. Leaves odd pinnate (-trifoliolate), petioled; leaflets paired, alternate, or irregularly arranged, entire; stipules free, persistent or caducous. Inflorescences axillary; bracts caducous. Calyx broad, bowl-shaped, lobes subequal or unequal, shorter or longer than tube; corolla early deciduous, reddish-orange, tinted with pink or salmon; stamens 10, diadelphous (9 + 1); styles glabrous. Fruits deflexed (-ascending or divergent), dehiscent (-indehiscent), oblong, subterete or tetragonal (-laterally compressed) in cross section, ovoid in one species, coriaceous. Seeds few to numerous.

KEY TO THE ALABAMA SPECIES OF *INDIGOFERA*

1. Stems hirsute or pilose; legumes hispid ..... 1. *I. hirsuta*
1. Stems glabrous or appressed strigose; legumes glabrous to strigose 2
   2. Leaflets, at least some, alternate or irregularly arranged on leafstalk; stipules 2-6 mm long; calyx 3-6 mm long ..... 2. *I. miniata*
   2. Leaflets opposite on leafstalk; stipules obsoluescent to 2 mm long; calyx 1-2 mm long 3
3. Legumes 5-10 mm long, ovoid, straight, indehiscent; seeds 2-3; stipules ..... obsoluescent; corolla 6-9 mm long ..... 3. *I. caroliniana*
3. Legumes 25-35 mm long, slightly falcate, dehiscent; seeds 3-10 or more; stipules 1-2 mm long; corolla 5-6 mm long ..... 4. *I. tinctoria*

1. *Indigofera hirsuta* Linnaeus, Sp. Pl. 751. 1753. [Figure 1a]
   Indigofera ferruginea Schumach & Thonner, Guin. Pl. 370. 1827.
   Indigofera fusca G. Don, Gen. Hist. 2: 211. 1832.
   Anila hirsuta (Linnaeus) Kuntze, Revis. Gen. Pl. 2: 939. 1891.

Herbs. Stems sprawling or erect, brownish hirsute or pilose. Leaves odd pinnate, petioles 3-8 cm long; leaflets 5-9 paired, elliptic to obovate, 2-4 cm long, pubescent both surfaces with subappressed hairs; stipules setaceous, to 1 cm long, persistent. Racemes 6-20 cm long, flowers crowded, pedunculate; pedicles 1 mm long. Calyx 3.5-5 mm long, lobes setaceous, longer than tube, bristly-plumose; corolla salmon to maroon, 6-7 mm long. Fruits 1.5-2.0 cm long, hispid, imbricate, deflexed, dehiscent, oblong, straight, turgid. Seeds numerous.

Native of Old World tropics, now pantropical, cultivated and established; introduced in southern United States.

Habitat and distribution in Alabama: roadsides, old fields, disturbed woodlands, waste areas; widely scattered in the southern one-fourth of the state (Fig. 1b).

Specimens examined. Henry County: *Lindsay Leverett 7*, 2 October 2008 (TROY), Mobile County: *Michel G. Lelong 9572*, 6 October 1976 (BRIT), Pike County: *Michael Woods 10210*, 22 November 2004 (TROY).

2. *Indigofera miniata* Ortega var. *leptosepala* (Nuttall *ex* Torrey & A. Gray) B. L. Turner, Field & Lab. 24: 104. 1956. [Figure 1c]

   Indigofera ornithopodioides Schlechtendal & Chamisso, Linnaea 5: 577. 1830.
   Indigofera leptosepala Nuttall *ex* Torrey & A. Gray, Fl. N. Amer. 1: 198. 1838.
   Indigofera cinerea Buckland, Proc. Acad. Phila. 1861: 451. 1861.
   Indigofera texana Buckland, Proc. Acad. Phila. 1861. 451. 1861.
   Anila leptosepala (Nuttall *ex* Torrey & A. Gray) Kuntze, Revis. Gen. Pl. 2: 939. 1891.

Herbs. Stems prostrate or ascending, greenish or cinereous, strigulose. Leaves odd pinnate, petioles 0.6-3 cm long; leaflets 5-9 opposite or irregularly arranged, cuneate-obovate to narrowly oblanceolate, 0.5-2.5 cm long; stipules subulate, 2-6 mm long, semipersistent. Flowers 3-numerous initially crowded, then loosening, shortly or well pedunculate; pedicels 1 mm long. Calyx 3-6 mm long, lobes longer than tube, subulate; corolla salmon-red (-pink, -orange), 8-12 mm long. Fruits 1.5-2.5 cm long, coriaceous, irregularly spreading or deflexed, dehiscent, oblong, straight, subterete. Seeds few to several.

Habitat and distribution in Alabama: ballast grounds in southwestern corner of the state (Fig. 1d).

Specimens examined. Mobile County: *Mohr s.n.*, September 1891 (UNA).

3. *Indigofera caroliniana* Miller, Gard. Dict., ed. 8. 1768. [Figure 2a]
Pithecellobium disperma Linnaeus, Syst. Nat., ed. 12. 3: 232. 1768.
Indigofera caroliniana Walter, Fl. Carol. 187. 1788, *non* Miller 1768.
Anila caroliniana Kuntze, Revis. Gen. Pl. 2: 939. 1891.

Herbs. Stems erect or ascending, slightly strigose. Leaves odd pinnate, petioles 2.5-7 cm long; leaflets (7-) 9-13 paired, obovate to oblanceolate, 0.8-2.5 cm long, inevidently stigulose both surfaces, pale below; stipules semipersistent. Racemes slender and lax, flowers numerous, shortly or well pedunculate; pedicles 1-2 mm long, in fruit to 3 mm long. Calyx 1.5-2 mm long, lobes deltate, ca. 0.5 mm long, much shorter than tube; corolla dark flesh-colored to ochroleucous (in fresh condition with rose, tan, and yellow), 6-9 mm long. Fruits 7-9 mm long, declined, persistent, indehiscent, ovoid or shortly oblong, compressed but turgid. Seeds 2-3.

Habitat and distribution in Alabama: pinelands, pine-palmetto, scrub oak communities, sandhills, and roadsides; scattered in the southern one-half of the state, mostly in southeastern corner (Fig. 2b).

Specimens examined. Baldwin County: *R. Kral 32617*, 19 August 1968 (BRIT), Barbour County: *John R. MacDonald 11291*, 27 May 1998 (UNA), Bullock County: *A.R. Diamond 13516*, 8 August 2002 (TROY), Coffee County: *Brian H. Martin 942*, 4 September 2000 (TROY), Crenshaw County: *A.R. Diamond 11379*, 23 August 1998 (AUA), Dale County: *Tiffany Pennington 820*, 27 June 2000 (TROY), Dallas County: *R. Kral 32872*, 22 August 1968 (BRIT), Henry County: *John R. MacDonald 12880*, 26 May 1999 (TROY), Houston County: *John R. MacDonald 19842*, 20 July 1997 (BRIT), Macon County: *D.A. Botts 202*, 2 July 1976 (AUA), Mobile County: *C. Mohr s.n.*, July (UNA), Montgomery County: *A.R. Diamond 12473*, 3 July 2001 (TROY), Pike County: *James A. Hall 56*, 3 July 2000 (TROY), Russell County: *R. Kral 62074*, 19 June 1978 (TROY).

4. *Indigofera tinctoria* Linnaeus, Sp. Pl. 751. 1753. [Figure 2c]
Indigofera anil Linnaeus var. *orthocarpa* de Candolle, Prodr. 2: 225. 1825.
Indigofera indica Lamarck, Encycl. 3: 245. 1789, *nom. illegit. et non* Miller 1768.

Anila tinctoria (Linnaeus) Kuntze, Revis. Gen. Pl. 1: 160. 1891.
Anila tinctoria (Linnaeus) Kuntze var. *normalis* Kuntze, Revis. Gen. Pl. 1: 160. 1891.
Anila tinctoria (Linnaeus) Kuntze var. *orthocarpa* (de Candolle) Kuntze, Revis. Gen. Pl. 1: 160. 1891.

Herbs. Stems erect or ascending (-sprawling), strigulose. Leaves odd pinnate, petioles 3-9 cm long; leaflets 9-15 paired, obovate or elliptic, 1-2.5 cm long, glabrous above; stipules ca. 2 mm long, caducous. Flowers closely disposed, initially subsessile, then pedunculate; pedicles 1-2 mm long. Calyx ca. 1.5 mm long, tube and lobes subequal; corolla reddish-orange, 5-6 mm long. Fruits crowded, 2.8-3.5 cm long, divergent to declined, deciduous, dehiscent, linear and slightly falcate or abruptly upturned at tip, subterete, usually submoniliform, thinly coriaceous, strigose. Seeds several to numerous.

Native of tropical Africa, now widely distributed in warm regions and tropics; introduced in southern United States.

Habitat and distribution in Alabama: ballast grounds in southwestern corner of the state (Fig. 2d).

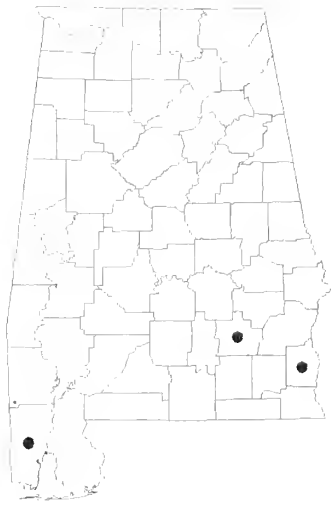
Specimens examined. Mobile County: *Mohr s.n.*, October 1869 (UNA).

DISCUSSION

In Alabama, *Indigofera* is a conspicuous taxon of pinelands, scrub oak communities, sandhills, roadsides, old fields, disturbed woodlands and urban waste areas.

The four taxa of *Indigofera* in this treatment are a combination of native and introduced species. *Indigofera caroliniana* Miller is based on a collection from "Carolina" and is the first North American species of the genus described (Miller, 1768). This taxon is endemic to the Southeastern United States and is known from all coastal states from North Carolina southwest to Louisiana (USDA, NRCS, 2010). Linnaeus (1753) described *I. hirsuta* based on a specimen collected from India. Presently, it has escaped cultivation in the United States and has been reported from Alabama, Florida, Georgia and South Carolina (USDA, NRCS, 2010). Although known from only three populations in Alabama, this taxon appears to be becoming more common in the southern section of the state, where two of the three known populations have been discovered in the past five years. Ortega (1798) described *I. miniata* based on a specimen collected from Cuba. This taxon is considered native to the United States and has been reported from Alabama, Arkansas, Georgia, Kansas, Louisiana, Oklahoma and Texas (USDA, NRCS, 2010). The Alabama collection is represented by *I. miniata* Ortega var. *leptosepala* (Nuttall ex Torrey & A. Gray) B. L. Turner. It is known from a single historical collection, September 1891, from ballast grounds in Mobile County. Although this taxon has not been reported from the state in 119 years, Alabama is in its natural range (Florida to Texas) and it possibly still occurs in the state. *Indigofera tinctoria* is the type of the genus and was described by

Linnaeus (1753) based on a specimen collected from India. In the United States, this taxon has escaped cultivation and has been collected in Alabama, Florida, North Carolina, South Carolina and Tennessee (USDA, NRCS, 2010). In Alabama, it is known from a single historical collection, October 1869, from ballast grounds in Mobile County. Since these ballast grounds have been developed, it is likely that this taxon is extirpated from the state.



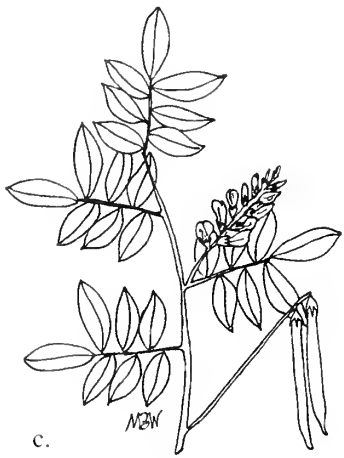


Figure 1. a) illustration of *Indigofera hirsuta*, b) distribution of *I. hirsuta*, c) illustration of *I. miniata*, d) distribution of *I. miniata*.

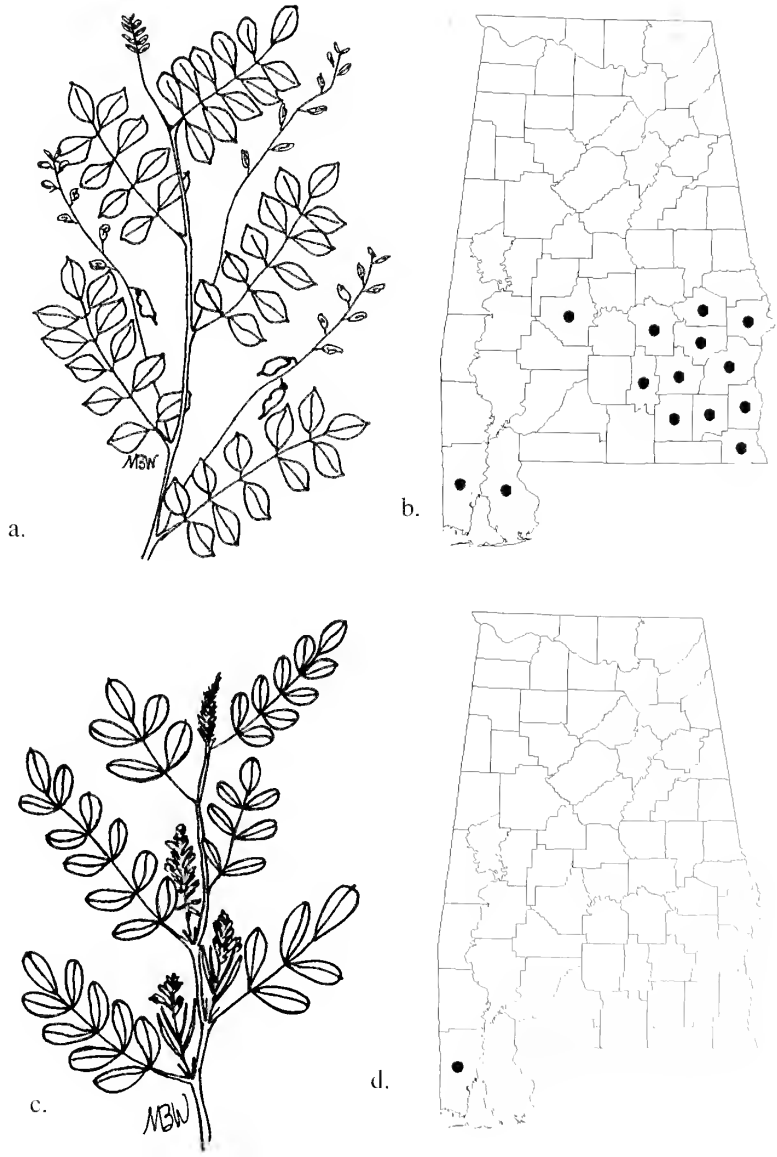


Figure 2. a) illustration of *Indigofera caroliniana*, b) distribution of *I. caroliniana*, c) illustration of *I. tinctoria*, d) distribution of *I. tinctoria*.

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